ACADEMIC PRESS

Journal of Invertebrate Pathology 79 (2002) 7–16

Journal of INVERTEBRATE PATHOLOGY

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Storage stability of *Anagrapha falcifera* nucleopolyhedrovirus in spray-dried formulations☆

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Received 30 January 2001; accepted 27 November 2001

Abstract

A multiply embedded nucleopolyhedrovirus isolated from *Anagrapha falcifera* (Kirby) (*Af*MNPV) can lose insecticidal activity during months of dry storage in ambient room conditions. We tested the spray-dried *Af*MNPV formulations after storage for up to 1 year at room temperatures for insecticidal activity against neonate *Trichoplusia ni* (Hübner). Experimental formulations were made using combinations of corn flours, lignin, and sucrose, and were selected based on previous work which demonstrated that these formulations resisted solar degradation in field experiments. Twelve experimental formulations (organized in three groups of four formulations) compared the effect of (1) the ratio of formulation ingredients (lignin and corn flour) to virus concentration, (2) different sources of lignin, or (3) different corn flours and sugar. Based on a single-dose plant assay with these 12 formulations, none of the formulations lost significant activity due to the drying process, when compared with the unformulated wet *Af*MNPV. Samples of the 12 dried formulations were stored at room (22 ± 3 °C) and refrigerated (4 °C) temperatures. Insecticidal activity (LC_{50}) was determined with a dosage–response assay for neonates fed on treated cotton-leaf disks. After 6 (or 9) and 12 months storage, refrigerated samples maintained insecticidal activity better than corresponding samples stored at room temperatures with LC_{50}s that averaged 2.0×10^6 polyhedral inclusion bodies per milliliter (pibs/ml) for refrigerated samples and 5.4×10^6 pibs/ml for samples stored at room temperatures. Compared with unformulated stock virus stored frozen, six formulations stored at room temperature and 10 formulations stored in the refrigerator did not lose significant insecticidal activity after 1 year based on overlapping 90% confidence intervals. Changing the ratio of virus to formulation ingredients did not provide a clear trend over the range of concentrations tested, and may be less important for shelf-life of virus activity compared with formulations made with different ingredients. Two of the four formulations made with different lignins were about 15 times less active after 1 year at room temperature compared with refrigerated samples, indicating that specific formulation ingredients can affect storage stability. Formulations that contained sugar generally maintained activity during storage better than formulations without sugar. Unformulated virus stock maintained insecticidal activity (ranged from 0.20 to 2.5×10^6 pibs/ml) better during storage than dried formulations with LC_{50}s that ranged from 0.39 to 27×10^6 pibs/ml. Unformulated virus stock, which is essentially a suspension of virus occlusion bodies in homogenized insect cadavers, did not lose activity when stored at refrigerated or room temperature. We believe that stability of *Af*MNPV insecticidal activity during storage as dry formulations is related to the general composition of the formulation and that sugar may play a critical role in maintaining insecticidal activity.

Keywords: *Anagrapha falcifera* nucleopolyhedrovirus; Baculovirus; Bioinsecticide; Shelf-life; Spray dry; Formulation; Lignin; Corn flour; Microencapsulation

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0022-2011/02/$ - see front matter
PII: S0022-2011(02)00005-8

1. Introduction

Insecticides based on microbial pathogens are an attractive alternative to chemical insecticides that can have an adverse environmental impact. The Baculoviridae are a promising family of viruses that may provide active agents for successful biopesticides because members of two groups, the nucleopolyhedroviruses (NPVs) and the granulosis viruses (GV), infect many important insect pests (Federici, 1999). The US EPA has six NPVs registered as biopesticide active ingredients (EPA.gov/pesticides/biopesticides/ai/all_ais.htm; updated September 26, 2001). *Anagrapha falcifera* multiply embedded NPV (*Af*MNPV), a variant of *Autographa californica* NPV (Chen et al., 1996; Federici and Hice, 1997), has been considered a promising candidate for development as a bioinsecticide for the control of lepidopteran pests of food and fiber crops (Vail et al., 1996) because its host spectrum includes several important pests.

Short residual activity, less than 1 day after field application, has been a major limitation for commercial development of NPVs (Burges and Jones, 1998). As with many other microbial agents, NPVs are inactivated by solar radiation (Burges and Jones, 1998; Ignoffo and Batzer, 1971; Jaques, 1971; McGuire et al., 2001; Shapiro, 1992). Formulations that encapsulate or attach the virus to a matrix have been used to minimize loss of activity due to solar radiation (Burges and Jones, 1998; Ignoffo and Garcia, 1996; Ignoffo and Batzer, 1971; Tamez-Guerra et al., 2000a).

Lignin is an abundant polymer found in all vascular plants that can be used to encapsulate virus particles. Lignin has an aromatic chemistry (moiety which acts as a UV chromophor) that provides protection of bioinsecticides from degradative processes initiated by solar radiation. In addition, the antioxidant property of lignin adds stability to normally unstable chemical pesticides to improve the efficacy of these chemical applications (DelliColli, 1980). Kraft lignin has received attention as an adjuvant in biopesticide formulations (Shasha et al., 1998), as well as for encapsulating *Bacillus thuringiensis* Berliner (Tamez-Guerra et al., 2000b) and baculoviruses (Tamez-Guerra et al., 2000a). Kraft lignin is water insoluble, however, a water-soluble lignin salt can be made by chemical modification with an alkalinizing agent such as sodium hydroxide. These modified kraft lignins have successfully encapsulated baculovirus occlusion bodies when processed by spray drying (Tamez-Guerra et al., 2000a). The result from this process is an encapsulated virus formulation (dry powder) with extended residual activity when tested in the laboratory and in the field (McGuire et al., 2001; Tamez-Guerra et al., 2000a). Development of these formulations may improve the potential for commercialization of baculoviruses as biological pesticides.

Another factor that limits commercialization of baculoviruses is poor storage stability (Wood and Granados, 1991). Three wettable-powder formulations of *Anticarsia gemmatalis* NPV (*Ag*MNPV) made with amorphous silica, attapulgite, and kaolinite maintained activity for 1 year of storage at room temperature, although one formulation made with bentonite lost activity (Medugno et al., 1997). Other dry formulations of some NPVs lost insecticidal activity during storage at room temperature (Lewis and Rollinson, 1978) and at cold temperature (Kaupp and Ebling, 1993). The mechanism responsible for this loss of activity during storage of dried virus has not been clearly described and may be the result of the formulation ingredients. Screening continues for stable formulations that can minimize loss of activity. The present study was conducted to evaluate the storage stability of spray-dried formulations of *Af*MNPV, in which the virus was encapsulated with chemically modified kraft lignin, corn flours, and sucrose. The initial insecticidal activity after production was determined, along with insecticidal activity after storage at refrigerated and room temperatures for up to 1 year.

2. Materials and methods

2.1. Insect colony

All assays used neonate cabbage looper, *Trichoplusia ni* (Hübner), from a colony maintained on artificial diet modified from Gardiner (1985) (Behle et al., 2000) at the USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL. Founders for this colony were obtained from USDA-ARS, Biological Control Insect Research Laboratory, Columbia, MO before 1995.

2.2. Virus source

Stocks of the baculovirus originally isolated from celery looper, *A. falcifera* (Kirby), were provided by Thermo Trilogy (formally Biosys, Columbia, MD). Virus stock contained 2.5×10^9 polyhedral inclusion bodies per milliliter (pibs/ml) and was stored frozen, at −70 °C until formulated.

2.3. Spray-dried formulations

In general, all formulations were made by dissolving modified lignin in water. The pH of the lignin solution was adjusted toward neutrality (9.0) with 85% lactic acid. Other ingredients were added followed by adding the virus. Finally, calcium chloride (10% solution) was added slowly. Materials for each of the formulations were mixed in water at 1–5% solids. These mixtures were typically viscous and propanol was added to thin the

dryer feed stock to minimize problems with the feed pump to the spray dryer. Before spray drying, the final pH of each formulation feed stock was adjusted to 7.5 with either a 10% KH_2PO_4 or NaOH solution. For each formulation, the virus concentration per gram of solids was calculated based on the count of the virus stock and the weights of the ingredient solids (Table 1), thus virus concentrations varied among formulations. To determine the insecticidal activity and storage stability of virus formulations, three groups of four formulations each were organized by relative ingredients (Table 1). Each of the groups compared variations on one "standard" experimental formulation based on a relative ratio of K-lignin to virus of 1:1 (K-lignin:NPV 1:1), which was made with potassium lignin and pregelatinized corn flour (PCF). Separate productions of this "standard" formulation were made for each group of formulations, indicated below.

For group 1, the standard K-lignin formulation was made with different virus concentrations. K-Lignin was prepared in our laboratory by mixing 90 g kraft lignin in 200 ml water and 11 g potassium hydroxide. This material was air-dried under the hood and ground to pass a 30-mesh sieve (Tamez-Guerra et al., 2000a). Formulations identified as (K-lignin:NPV) 1:1, 2:1, and 3:1 contained 2.5, 1.25, and 0.6×10^9 pibs/g, respectively. The fourth formulation in this group was K-lignin + sugar and was selected based on positive results when used to formulate *B. thuringiensis* (Tamez-Guerra et al., 2000b). The K-lignin + sugar formulation was made with K-lignin, PCF, sucrose, and lactic acid with a 2:1 ratio of formulation ingredients to virus (1.25×10^9 pibs/g).

For group 2, formulation ingredients were selected to compare different sources of soluble lignin for making the standard K-lignin formulation, described above. Lignin variations included: Na-lignin #1 = Indulin C, commercial production process (Westvaco, North Charleston, SC), and laboratory produced Na-lignin #2 = Indulin C, and K-lignin prepared by Westvaco.

Group 3 included formulations with different ratios of K-lignin to corn flours and sugar. The standard formulation (K-lignin:PCF 1:1) described above was included. A second formulation (K-lignin:flour 2:1) had twice as much lignin relative to the amount of PCF. The third lignin formulation (K-lignin + NCF + sugar) contained nixtamalized corn flour (NCF) in addition to PCF and sucrose. The fourth formulation (NCF + sugar) contained both corn flours and sucrose but did not contain lignin.

2.4. Spray drying

All spray-dried formulations were made using a Niro Atomizer Spray Dryer (Niro Atomizer, Columbia, MD). Spray-drying conditions were selected based on previous tests with *B. thuringiensis* (Tamez-Guerra et al., 1996) and baculovirus (Tamez-Guerra et al., 2000b). Spray-drying conditions were 100 °C inlet temperature, 65–70 °C outlet temperature, 5.6 kg/cm^2 air pressure for the atomizing wheel, and 8 ml/min product feed rate.

Table 1
Ingredients used to make spray-dried formulations of *Af*MNPV for storage stability experiments

Formulation	Lignin (g)	PCF[a] (g)	Sucrose (g)	$CaCl_2$ (g)	Water (ml)	2-Propanol (ml)	*Af*MNPV[b] (pibs × 10^9/g formulation)
Group 1							
K-Lignin:NPV 1:1 (standard)	5.0[c]	5		1	700	100	2.5
K-Lignin:NPV 2:1	10.b[c]	10		2	800	200	1.2
K-Lignin:NPV 3:1	20.0[c]	20		4	1200	300	0.62
K-Lignin + sugar:NPV 2:1	5.0[c]	5	10	1	400	100	1.2
Group 2							
K-Lignin (standard)	5.0[c]	5		1	700	100	2.5
K-Lignin (Westvaco)	5.0[d]	5		1	700	100	2.5
Na-Lignin (Westvaco #1)	5[e]	5		1	700	100	2.5
Na-Lignin (Westvaco #2)	5.0[f]	5		1	700	100	2.5
Group 3							
K-Lignin:PCF 1:1 (standard)	15.0[c]	15		3	1200	300	2.5
K-Lignin:PCF 2:l	20.0[c]	10		4	1000	200	8.3
K-Lignin + NCF[g] + sugar	2.5[c]	2.5	5	1.2	700	100	1.6
NCF[g] + sugar		5	5		700	100	1.4

[a] Pregelatinized corn flour, Flour 965; Illinois Central Mills, Paris, IL.
[b] *Af*MNPV stock contained 2.5×10^9 pibs/ml provided by Thermo Trilogy, Columbia, MD.
[c] Potassium lignin made at USDA in Peoria, IL, see text for details.
[d] Potassium lignin (Westvaco).
[e] Sodium lignin #1 (Westvaco).
[f] Sodium lignin #2 (Westvaco).
[g] Nixtamalyzed corn flour, Maseca; Guadalupe, N.L. Mexico included at an amount equal to the amount of PCF added to this formulation in addition to 0.05 ml lactic acid (85%, Fisher, Pittsburg, PA) and 2.5 ml corn oil (Mazola, CPC International, Englewood Cliffs, NJ).

2.5. Storage conditions, sampling, and evaluations

Before storage, initial insecticidal activity of formulations was compared with unformulated virus using a single-dosage plant assay, described below. Then, each formulation was divided into two samples of 5 g each and placed separately in zip-seal plastic bags. One sample of each formulation was stored in a drawer at room temperature (22 + 3 °C) and the other in a refrigerator (4 °C). Insecticidal activity was determined for subsamples removed from these storage bags. Samples of the corresponding unformulated viruses were placed in a 1 ml Eppendorf plastic tube for storage with the dried formulations.

2.6. Assays for insecticidal activity

Two leaf-feeding assay procedures were used to determine the relative insecticidal activity among *Af*MNPV formulations. A single-dosage assay was used to determine initial insecticidal activity after production and a dosage–response assay was used to determine insecticidal activity after storage. For both cotton-leaf-feeding assays, "DES 119" cotton was grown in a glasshouse for 4–6 weeks. Leaves from these plants were rinsed with tap water before use in assays. Leaf disks (33-mm diameter) were cut from rinsed cotton leaves and were placed individually in a plastic 50 × 9 mm Petri dish with a filter paper (42.5 mm #1, Whatman, Whatman International, Maidstone, England) to absorb excess moisture. Five dishes were prepared for each treatment. Five leaf disks that did not receive a virus treatment were included with each assay as a no-treatment control to provide an indication of handling mortality.

Single-dosage cotton-leaf bioassay. Before storing the virus formulations, each was subjected to a single-dosage cotton-leaf bioassay to verify insecticidal activity after production. Each virus treatment was mixed in water at a concentration of 5×10^6 pibs/ml, a concentration known to cause about 85% mortality for unformulated virus when using the following procedures. A 100 μl sample of a virus treatment was pipetted onto the upper leaf surface of each leaf disk and was spread over the surface of the leaf disk with a glass rod. Once the treatments had air-dried, 10 newly hatched *T. ni* were placed into each dish and incubated at 27 °C for a 22–24 h feeding period. Larvae did not consume the entire leaf. After feeding, six live larvae from each of five dishes for each treatment were transferred to one of the 30 individual cups (37 ml, T-125, Solo Cup, Urbana, IL) containing artificial diet, totaling 30 larvae per treatment. Transferring the larvae to individual cups prevented cannibalism and horizontal virus transmission. These were incubated for an additional 6 days. Percentage mortality was recorded 7 days after larvae were exposed to treated leaf disks. This procedure was replicated by repeating the procedure on three sequential days. Mortality data were subjected to analysis of variance, and means were separated using a protected least significant difference (LSD) (Statistix, 1996).

Dosage–response cotton-leaf assay. The dosage–response assay was used to determine the insecticidal activity of each formulation after storage. Five concentrations for each formulation were prepared by 1:2 serial dilutions of virus from 1×10^7 to 1.2×10^5 pibs/ml. Five leaf disks were treated and larvae were exposed to treated leaf disks, as described above, to provide 30 exposed larvae for each formulation concentration. Virus samples were tested after 9 and 12 months storage for the formulations of group 1 and after 6 and 12 months for formulations in groups 2 and 3. The corresponding unformulated (wet) virus stock stored frozen (−70 °C) was assayed as a control treatment after 12 months storage. Dosage–response data were analyzed using POLO-PC (LeOra, 1987) based on Finney (1971) to determine the LC_{50} and 90% confidence limits for each treatment. Experimental formulations were considered significantly different when 90% confidence limits for the LC_{50} did not overlap. We used 90% confidence limits for comparison because the "*g*" calculation for some data was sufficiently large to prevent calculation of a 95% limit by the POLO-PC program and we want to report a consistent method for comparison.

Moisture content analysis. Moisture content of each formulation (1 g sample) was determined using a Mark 2 Moisture Analyzer (Onmimark, Temple, AZ). Formulations were sampled for moisture content after 1-year storage at room temperature.

2.7. Additonal statistics

A paired *t* test was used to compare storage temperatures (Proc Means; SAS, 1989). For paired LC_{50} data, analysis consisted of comparing the difference between LC_{50}s with zero [H_0: (LC_{50} refrigerated) − (LC_{50} room temperature) = 0]. Both raw data and log transformed data were analyzed for this paired test. Additionally, simple linear regression analysis (Proc REG; SAS, 1989) was used to relate the insecticidal activity of dried samples stored at room temperature to the moisture content of those samples.

3. Results

3.1. Initial insecticidal activity of *Af*MNPV formulations

Spray-dried formulations of *Af*MNPV did not show significant loss of activity due to the spray-drying process. Before storage, each of the formulations provided mortalities from the single-dosage cotton-leaf assay that

Table 2
Effect of spray drying on insecticidal activity of *Af*MNPV against *Trichoplusia ni* using a single dose bioassay[a]

Group 1	Mortality (%)	Group 2	Mortality (%)	Group 3	Mortality (%)
K-Lignin:NPV 1:1 (standard)	68.8 bc	K-Lignin (standard)	75.0 b	K-Lignin:PCF 1:1 (standard)	83.7 bc
K-Lignin:NPV 2:1	80.0 c	K-Lignin	84.8 b	K-Lignin:PCF 2:1	82.5 bc
K-Lignin:NPV 3:1	77.7 c	Na-Lignin	89.8 b	K-Lignin + NCF + sugar	70.8 b
K-Lignin + sugar:NPV 2:1	96.6 d	Na-Lignin	79.8 b	NCF + sugar	86.9 c
Unformulated	60.0 b	Unformulated	84.8 b	Unformulated	78.9 bc
Control	4.4 a	Control	1.2 a	Control	1.2 a
F (5,17)	63.5		26		64
CVC	12.3		20.4		12.6

[a] Refer to Table 1 for formulation composition, single dose bioassay, using (1×10^6 pibs/ml). PCF, pregelatinized corn flour; NCF, nixtamalized corn flour; CVC, critical value for comparison for each group. Means in a column followed by the same letter are not significantly different, LSD, $P = 0.05$.

were equal to or greater than the corresponding unformulated treatment (Table 2). Three dried formulations (one made with sugar and two made without sugar) in group 1 had significantly ($F_{5,17} = 63.5$, $P < 0.05$) higher activity than the unformulated treatment. In groups 2 and 3, treatments (including two formulations made with sugar) were not significantly ($F_{5,17} = 26.0$, $P > 0.05$ and $F_{5,17} = 64.0$, $P > 0.05$, respectively) different from their respective unformulated treatments.

Few differences were observed among the dried formulations in each respective group. Formulations made with sugar need specific attention because of the potential for sugar to induce feeding on treatment residue, effectively increasing the exposure of larvae to virus. In group 1, the K-lignin + sugar formulation had significantly higher mortality than all other formulations tested. In group 3, the NCF + sugar formulation (without lignin) caused higher mortality than the K-lignin + NCF + sugar formulation. The mortality for the K-lignin (standard) formulation (78.2%) averaged from all three groups was not significantly different ($F_{5,17} = 2.86$, $P = 0.0628$) from the averaged mortality for the unformulated *Af*MNPV (74.5%), providing additional evidence indicating that spray drying did not reduce initial insecticidal activity.

3.2. Storage stability

Unformulated virus stock stored at −70 °C for 12 months showed no loss of activity. The LC_{50} for three unformulated virus stock samples averaged 1.14×10^6 pibs/ml (SD = 0.49) with an average slope of 1.07 (SD = 0.06, range from 1.0 to 1.1). Individually, the LC_{50}s were 9.1×10^5 pibs/ml for group 1, 1.7×10^6 pibs/ml for group 2, and 8.1×10^5 pibs/ml for group 3. Results comparing individual assays showed that insecticidal activity was not significantly different among these samples based on overlapping confidence limits indicating that the virus stocks were relatively consistent among groups of formulations.

Unformulated virus did not lose insecticidal activity when stored at refrigeration and room temperatures. For the six assays of unformulated virus (Tables 3–5), samples stored between 6 and 12 months under refrigeration averaged an LC_{50} of 1.14×10^6 pibs/ml (SD = 0.57) and samples stored under room conditions averaged 1.15×10^6 pibs/ml (SD = 0.78). Based on the paired *t* test, the mean LC_{50}s for unformulated virus stock samples were not different between room temperature and refrigerated stored samples. Likewise, the mean LC_{50} for unformulated virus stored at room temperature for 12 months was not significantly different ($n = 3$, raw data: -0.21×10^6 pibs/ml, $t = -0.40$, $P = 0.728$; log transformed: −0.23, $t = -0.48$, $P = 0.679$) from the mean LC_{50} for the stock virus stored frozen.

Unlike the unformulated samples, the spray-dried samples stored at room temperature had higher LC_{50} values compared with refrigerated samples indicating a greater loss of activity when stored at a higher

Table 3
LC_{50} and 90% confidence limits of spray-dried formulations of *Af*MNPV after storage (group 1)

Formulations	LC_{50} ($\times 10^6$ pibs/ml) after 9 months		LC_{50} ($\times 10^6$ pibs/ml) after 12 months	
	Room temperature	Refrigeration	Room temperature	Refrigeration
Lignin:NPV 1:1 (standard)	4.6 (3.2–6.5)	2.1 (1.3–3.3)	8.3 (3.6–24)[a]	0.59 (0.38–0.94)[a]
Lignin:NPV 2:1	4.3 (2.7–6.6)	2.8 (2.0–4.4)[a]	12.8 (3.9–21)[a]	3.00 (1.5–43)[a]
Lignin:NPV 3:1	15.0 (9.9–28)[a]	0.63 (0.29–5.9)	1.6 (0.9–3.9)[a]	0.84 (0.59–4.9)[a]
Lignin + sugar:NPV 1.5:1	6.4 (4.8–8.7)[a]	0.84 (0.42–1.3)	0.65 (0.4–2.0)	0.39 (0.27–0.81)
Unformulated	1.2 (0.42–3.6)	0.94 (0.64–1.3)	0.70 (0.46–0.96)	0.20 (0.12–0.29)

Numbers in parentheses are the lower and upper confidence limit values, Probit, POLO-PC.
[a] Formulation sample was significantly different than the related unformulated *Af*MNPV based on 90% confidence limits that do not overlap.

Table 4
LC_{50} and 90% confidence limits of spray-dried formulations of *Af*MNPV after storage (group 2)

Formulations	LC_{50} ($\times 10^6$ pibs/ml) after 6 months		LC_{50} ($\times 10^6$ pibs/ml) after 12 months	
	Room temperature	Refrigeration	Room temperature	Refrigeration
K-Lignin (USDA) (standard)	8.9 (5.6–21)[a]	2.3 (1.7–3.1)	21.0 (14–31)[a]	1.4 (0.98–1.9)
K-Lignin (Westvaco)	2.7 (2.1–3.5)[a]	1.2 (0.76–1.7)	5.1 (3.0–10)[a]	0.9 (0.58–1.5)
Na-Lignin (Westvaco #1)	1.6 (0.61–30)	1.3 (0.52–2.0)	4.2 (2.7–10)[a]	1.2 (0.76–4.1)
Na-Lignin (Westvaco #2)	7.3 (4.3–22)[a]	1.4 (0.94–1.9)	27.0 (6.2–88)[a]	2.6 (2.0–4.1)
Unformulated	0.4 (0.023–0.85)	1.2 (0.64–1.3)	0.6 (0.40–0.83)	1.2 (0.72–2.9)

Numbers in parentheses are the lower and upper confidence limit values, Probit, POLO-PC.
[a] Formulation sample was significantly different than the related unformulated *Af*MNPV based on 90% confidence limits that do not overlap.

Table 5
LC_{50} and 90% confidence limits of spray-dried formulations of *Af*MNPV after storage (group 3)

Formulations	LC_{50} ($\times 10^6$ pibs/ml) after 6 months		LC_{50} ($\times 10^6$ pibs/ml) after 12 months	
	Room temperature	Refrigeration	Room temperature	Refrigeration
Lignin:PCF 1:1 (standard)	4.0 (3.3–5.0)[a]	2.0 (1.2–3.0)	16.2 (4.2–230)	2.8 (1.8–7.7)
Lignin:PCF 2:l	4.0 (2.2–9.9)	1.4 (1.0–1.9)	3.4 (1.9–7.3)	10.0 (6.7–21)[a]
Lignin + NCF + sugar	2.0 (1.3–3.0)	1.6 (1.2–2.0)	2.5 (1.4–6.1)	8.0 (2.4–500)[a]
NCF + sugar	1.7 (0.98–2.7)	3.6 (2.8–5.0)[a]	4.4 (2.4–8.1)	1.1 (0.6–3.9)
Unformulated	2.5 (2.0–3.2)	1.9 (1.5–2.5)	1.5 (0.72–5.6)	1.1 (0.60–2.2)

Numbers in parentheses are the lower and upper confidence limit values, Probit, POLO-PC.
[a] Formulation sample was significantly different than the related unformulated *Af*MNPV based on 90% confidence limits that do not overlap.

temperature. A paired t test including all 6-, 9-, and 12-month data for all formulations showed that the mean LC_{50} for samples stored at room temperature was significantly greater ($+3.86 \times 10^6$ pibs/ml, $t = 3.13$, $P = 0.004$) than that for refrigerated samples. For the K-lignin + PCF 1:1 treatment, which was included in all three groups, the samples stored at room temperature averaged a significantly greater LC_{50} (raw data 8.63×10^6 pibs/ml, $t = 3.12$, $P = 0.026$; log transformed 1.66, $t = 4.16$, $P = 0.006$) compared with refrigerated samples.

Formulations in group 1 were intended to compare formulations with different concentrations of virus in the spray-dried product for shelf-life. Unfortunately, the results were inconsistent among samples and did not indicate obvious trends relating virus concentrations with changes in insecticidal activity after storage. For example, the lignin + PCF 3:1 formulation had a significantly higher LC_{50} at the 9-month sample than it did after 12 months storage at room temperature (Table 3). After 12 months storage, results showed that formulations with lignin + PCF generally lost activity compared with the unformulated virus. The lignin:NPV 3:1 formulation showed lower insecticidal activity (higher LC_{50}) compared with other formulations after 6 months storage at room temperature. The lignin:NPV 2:1 formulation showed lower insecticidal activity compared with lignin + sugar:NPV 1.5:1 formulation after 9 months under refrigeration. The lignin + sugar:NPV 1.5:1 formulation showed the highest insecticidal activity compared with other formulations after 12 months storage at room temperature. Both lignin:NPV 1:1 and lignin + sugar:NPV 1.5:1 formulations showed a higher activity compared with formulation lignin:NPV 2:1 after 12 months refrigerated storage.

For formulations in group 2, results were more consistent than for the formulations in group 1. Similar results were observed for samples stored for 6 and 12 months, with differences being more pronounced with longer storage at room temperature (Table 4). When stored at room temperature for 12 months, all formulations with different lignin salts showed reduced activity compared with unformulated virus, K-lignin (Westvaco) and Na-lignin (Westvaco #1) formulations showed higher insecticidal activity compared with K-lignin (USDA) or Na-lignin (Westvaco #2) formulations.

In group 3, two formulations with different proportions of lignin versus PCF, and NCF + sugar with or without lignin were tested for storage stability. Two formulations with higher concentrations of lignin (lignin:PCF 2:1 and lignin + NCF + sugar) had lesser insecticidal activity after 1 year of refrigerated storage compared with the unformulated virus sample (Table 5). The lignin:PCF 1:1 formulation showed lower insecticidal activity compared with lignin + NCF + sugar, NCF + sugar, and unformulated samples after 6 months storage at room temperature. In general, these results show that dry formulations lose more insecticidal activity when stored at higher temperatures, and that the two formulations with sugar (average $LC_{50} = 3.11 \times 10^6$ pibs/ml, $n = 8$, SD = 2.26) tend to maintain insecticidal activity compared to two formulations made without sugar (average $LC_{50} = 5.47 \times 10^6$ pibs/ml, $n = 8$, SD = 5.07).

Table 6
Moisture content of spray-dried formulations initial and after 1-year storage at room temperature in zip-seal plastic bags

Formulations[a]	Moisture (%)	
	Initial	After 1 year
Group 1		
Lignin:NPV 1:1 (standard)	7.1	8.2
Lignin:NPV 2:1	7.5	8.2
Lignin:NPV 3:1	7.9	9.6
Lignin + sugar:NPV 1.5:1	4.5	5.6
Group 2		
K-Lignin (USDA) (standard)	7.9	11.6
K-Lignin (Westvaco)	7.5	11
Na-Lignin (Westvaco #1)	6.9	8.9
Na-Lignin (Westvaco #2)	7.3	11.1
Group 3		
Lignin:PCF 1:1 (standard)	7.7	10.5
Lignin:PCF 2:1	7.4	9.1
Lignin + NCF + sugar	5.7	7.4
NCF + sugar	5	6.5

[a] Refer to Table 1 for formulation composition. PCF, pregelatinized corn flour; NCF, nixtamalized corn flour.

3.3. Moisture content

Analysis after 12 months storage in plastic bags at room temperature showed that the samples had between 5% and 12% moisture (Table 6), and all samples gained moisture during storage. Overall, formulations that contained sucrose had the lowest moisture, with 5.6%, 7.4%, and 6.5% for lignin + sugar:NVP 1.5:1 (group 1), lignin + NCF + sugar (group 3), and NCF + sugar (no lignin, group 3) formulations, respectively. The moisture content of the "standard" formulation in all three groups was between 8.2% and 11.6% after 12 months storage at room temperature. Except for the formulation based on Na-lignin (Westvaco #1), all other formulations from group 2 showed moisture content slightly higher than 10%. In general, when formulations were stored in plastic bags all samples gained moisture, with an average of 2.05%. Linear regression analysis comparing moisture content to LC_{50} values [slope $= (0.12 \times 10^6$ pibs/ml)/(1% moisture), $P = 0.07$] indicated that the moisture content accounted for 29% of the variability ($R^2 = 0.29$) in the LC_{50} values after 1-year storage at room temperature. This trend would indicate that a loss of insecticidal activity is related to a higher moisture content in the spray-dried formulations and that additional efforts to reduce and maintain low moisture may benefit storage stability.

4. Discussion

The use of baculoviruses for insect biological control is an attractive pest control option due to their high virulence to specific insect pests and low hazard to humans and the environment. The ability to formulate virus for application with available spray equipment makes viruses more attractive as biological control agents (McNitt et al., 1995). Commercially, dried formulations have an advantage over liquid products for storage and handling because of reduced weight and package size. Disadvantages of dry formulations include dustiness, inhalation risk, bulk, and a need to keep product dry (Seaman, 1990). Yet, storage stability of virus remains an important issue for commercialization of any formulation. The length of storage required for each product depends on the specific claims on the label registered with the Environmental Protection Agency (EPA). Activity of the product cannot vary significantly (as stated by EPA regulations) when it is sold to the public. Longer storage stability (measured in years) is obviously desirable, but shorter storage stability (measured in months) can be addressed with expiration dates for the product. The data in this manuscript would suggest that these virus formulations would fit the shorter storage stability group.

Lyophilization (freeze-drying) has been the most common method for stabilizing virus (Burges and Jones, 1998), although this procedure is considered expensive and dry formulations may lose insecticidal activity during storage at room temperature (Lewis and Rollinson, 1978). Kaupp and Ebling (1993) reported on the effect of mechanical processing and storage of Virtuss, an NPV product used in Canada since 1975, for the control of whitemarked tussock moth, *Orgia leucostigma* (J.E. Smith), in forests. Virtuss is produced by lyophilization of the virus-infected dead larvae, milling to a fine powder, and storage in plastic bags at 4 °C until use. The potency of Virtuss was not affected in the production process, but a 50% loss of insecticidal activity was observed after storage for 2 years (Kaupp and Ebling, 1993). Insecticidal activity of *Lymantria dispar* (Linnaeus) NPV stored at 4 °C was slightly reduced after 10 months for a freeze-dried formulation, and after 3 months for air-dried formulations (Lewis and Rollinson, 1978). In contrast, Medugno et al. (1997) reported that three of four *Ag*MNPV formulations spray-dried with different clays maintained activity after storage for 1 year. Our results concur with a loss of activity during dry storage, demonstrating that many of our dried formulations lost insecticidal activity when stored refrigerated and at room temperature. Thus, simply drying *Af*MNPV may not provide adequate protection for shelf-life stability and contributes to the negative aspects further limiting commercialization of *Af*MNPV-based biopesticides.

Another method of stabilization is to encapsulate the virus in a matrix. Ignoffo et al. (1991) suggested that viruses encapsulated with cornstarch to make granular formulations could reduce the production costs.

Solubilized cornstarch was mixed with the *Heliothis* NPV, dried, then ground to a fine powder to produce particles with virus both on the surface and throughout the interior of each particle. Although this process produced a formulation that provided protection from solar radiation (Ignoffo et al., 1991), the virus was not truly microencapsulated, which provides each pib with its own protective coating.

The spray-drying technique used to formulate NPV described in this manuscript produces microencapsulated pibs. Pib encapsulation was verified by transmission and scanning electron microscopy (Tamez-Guerra et al., 2000a). In this study, lignin- and corn flour-based formulations were selected based on previous research with *B. thuringiensis* and *Af*MNPV (Tamez-Guerra et al., 2000a,b). The lignin formulations have demonstrated extended residual activity in laboratory experiments after simulated rain and sunlight exposures (Tamez-Guerra et al., 2000a), and in field tests (McGuire et al., 2001).

Spray drying may be a suitable production technique for some baculoviruses because it allows selection from a variety of ingredients to provide improved field performance and potentially longer storage stability. The first report of spray drying to make NPV formulations used *Heliothis* NPV, where agglomerates of inclusion bodies were spray-dried with different inert ingredients into wettable-powder formulations (Ignoffo et al., 1976). When testing *Helicoverpa zea* NPV formulations, Bull et al. (1976) reported that TiO_2 and carbon black were significantly better than other ingredients for maintaining stability and residual activity when exposed to UV light. Spray-dried wettable-powder formulations of *Ag*MNPV made with inert clays and amorphous silica (Medugno et al., 1997) showed the activity and physical performance of virus formulated with attapulgite amorphous silica were not affected after storage of ambient temperature for up to 1 year. In contrast, activity was lost and physical quality degraded with a kaolinite-based formulation (Medugno et al., 1997). These results demonstrate the need to consider multiple product characteristics when selecting formulation ingredients.

The comparisons of the dosage–response data presented herein are based on 90% confidence limits, which are narrower than 95% limits. Thus, we report more differences among the samples than would be reported if 95% limits were used. The POLO-PC program did not provide 95% limits for each sample because of variability in the data that resulted in the calculation of an index of significance for potency estimation, g, that was greater than 0.49. When this limit was exceeded for the 95% level, confidence limits for these data were not reported by the program. Limitations on time and materials prevented repeating assays to verify data when unusual or variable results were obtained. The 90% limits were obtained for all the data and were reported for comparisons.

This is the first report on storage stability of *Af*MNPV spray-dried formulations. The results of insecticidal activity after storage of experimental formulations with different proportions of virus and total solids (group 1) did not show a clear relationship between pib concentration in the formulations and loss of insecticidal activity (Table 3). In general, the virus activity was more stable when sugar was added to the formulation (Tables 3 and 5). When potassium and sodium lignin salts were compared, the formulations made with lignin salts from Westvaco maintained virus activity during storage better than the potassium lignin made at the USDA laboratory (Table 4). The *Af*MNPV in all the formulations of group 2 lost activity compared with the unformulated virus. For formulations made with different proportions of ingredients (group 3), the results were inconsistent although each of the dried formulations lost activity relative to the wet unformulated samples (Table 5). The NCF + sugar formulation (made with sugar and without lignin) maintained insecticidal activity for up to 1 year at room temperature without losing insecticidal activity (Table 5).

In general, we observed that formulations made with sucrose had a longer shelf-life than formulations without sugar (Tables 3 and 5). Sugars are used in many ways as a preservative as indicated by supporting literature, which is too extensive to begin to present in this manuscript except for a few examples. Increasing sugar (sucrose and others) concentrations in solutions reduce water activity sufficiently to prevent microbial growth (Brocklehurst et al., 1995). Sugars (predominantly trehalose) have been studied for effects in preserving the structure and function of biological systems during dehydration and subsequent storage (Sun and Davidson, 1998). Leslie et al. (1995) found that the addition of sucrose to *Escherichia coli* and *B. thuringiensis* maintained the viability of the samples when they were freeze-dried by protecting the membranes from detrimental effects of drying. Also, sucrose has been shown to protect non-occluded *Af*MNPV while air-drying (Behle et al., 2000). Thus, sugar may be important for protecting the virus during drying and storage in numerous ways that benefit shelf-life.

It must be noted that formulation ingredients such as flour, sugar, and oil often act as feeding stimulants (phagostimulants) and may apparently increase the insecticidal activity of some formulations. Such a situation could mask a loss of insecticidal activity by inducing test insects to selectively feed on treatment residue, effectively increasing their exposure. Several spray additives have been marketed to improve the efficacy of *B. thuringiensis* by increasing the palatability of spray residue. Farrar and Ridgway (1994) tested six hagostimulants

with larvae of six lepidoptera species (not including *T. ni*) and showed small differences for the amount of feeding between treated and non-treated leaf tissue in two choice tests. Previous experiments with freshly prepared formulations similar to the formulations tested here did not give indications of enhanced initial activity to *Spodopters frugiperda* (J.E. Smith) or *H. zea* (Boddie) when compared with unformulated virus in diet overlay assays (Tamez-Guerra et al., 2000a). Treatment concentrations in the assays presented here were typically mixed at less than 1% w/v of formulation, depending on the pib concentration in the formulation, and were less than the 5% concentrations tested by Farrar and Ridgway (1994). In contrast to stimulating larval feeding, calcium chloride (Gillespie et al., 1994) and higher lignin content in leaves (imparting greater leaf toughness) (Dowd and Lagrimini, 1997) have been suggested as feeding deterrents. We did not observe a dramatic increase or decrease in initial insecticidal activity to suggest an advantage for some formulations in terms of induced or reduced feeding on treatment residue. Admittedly, the effects of formulation palatability cannot be eliminated from consideration. Yet, based on the amount of material applied and additional experiences not reported here, we feel the results presented herein reflect actual insecticidal activity with little or no alteration due to differential feeding among formulation treatments applied to leaf tissue.

Moisture content seemed to play a role in storage stability of virus formulations. When comparing different kinds of lignin (group 2), the formulation made with Westvaco Na-lignin #1 had the lowest moisture content and the highest insecticidal activity after storage at room temperature (Table 4). Among these formulations, the Westvaco Na-lignin #1 had a moisture content of 8.9% compared with 11.6% for USDA's K-lignin, 11.0% for Westvaco's K-lignin, and 11.1% for Westvaco's Na-lignin #2. Similarly, when looking at all three groups, the formulations in group 2 had higher LC_{50}s and averaged 11.6% moisture versus 8.2% and 10.5% for groups 1 and 3, respectively. We found that 5 of 12 spray-dried formulations had above 10% moisture after storage for 1 year at room temperature (Table 6). It is unclear if the moisture content of these samples is an indicator of the loss of activity or a contributing factor to insecticidal degradation.

The results presented here document the storage stability of baculovirus formulations made by spray drying with lignin. These formulations generally demonstrated inconsistent results. Better storage stability of dry formulations is still an issue for viruses to be considered as a viable commercial bioinsecticide. Additional work concerning ingredient selection, processing techniques, storage containers, and moisture content need to be completed to improve storage stability of these formulations.

Acknowledgments

We sincerely thank Erica Bailey, Holly Goebel, Monica Wetzel, Jennifer Petersen, and Ruby Rodriguez for their assistance while conducting these assays. We also thank Michele Giovanini for comments on early drafts of the manuscript and Debra Palmquist for assistance with statistical analyses.

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